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Combined Physical and Genetic Map of the *Pseudomonas putida* KT2440 Chromosome

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A combined physical and genetic map of the *Pseudomonas putida* KT2440 genome was constructed from data obtained by pulsed-field gel electrophoresis techniques (PFGE) and Southern hybridization. Circular genome size was estimated at 6.0 Mb by adding the sizes of 19 *Swa*I, 9 *Pme*I, 6 *Pac*I, and 6 I-*Ceu*I fragments. A complete physical map was achieved by combining the results of (i) analysis of PFGE of the DNA fragments resulting from digestion of the whole genome with *Pme*I, *Swa*I, I-*Ceu*I, and *Pac*I as well as double digestion with combinations of these enzymes and (ii) Southern hybridization analysis of the whole wild-type genome digested with different enzymes and hybridized against a series of probes obtained as cloned genes from different pseudomonads of rRNA group I and *Escherichia coli*, as *P. putida* DNA obtained by PCR amplification based on sequences deposited at the GenBank database, and by labeling of macrorestriction fragments of the *P. putida* genome eluted from agarose gels. As an alternative, 10 random mini-Tn*5*-Km mutants of *P. putida* KT2440 were used as a source of DNA, and the band carrying the mini-Tn*5* in each mutant was identified after PFGE of a series of complete chromosomal digestions and hybridization with the kanamycin resistance gene of the mini-Tn*5* as a probe. We established a circular genome map with an average resolution of 160 kb. Among the 63 genes located on the genetic map were key markers such as *oriC*, 6 *rrn* loci (*rrnA* to *-F*), *recA*, *ftsZ*, *rpoS*, *rpoD*, *rpoN*, and *gyrB*; auxotrophic markers; and catabolic genes for the metabolism of aromatic compounds. The genetic map of *P. putida* KT2440 was compared to those of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas fluorescens* SBW25. The chromosomal backbone revealed some similarity in gene clustering among the three pseudomonads but differences in physical organization, probably as a result of intraspecific rearrangements.

Pseudomonas putida is a member of rRNA group I of the genus *Pseudomonas*. This species is able to colonize many different niches, including soil, freshwater, and the surfaces of living organisms (e.g., the roots of agriculturally important plants) (9, 37, 42, 49, 60). A number of different strains have been isolated from these niches, and a relevant property of all of them is the ability to metabolize a wide range of biogenic and xenobiotic compounds. *P. putida* mt-2 was isolated from soils by virtue of its ability to use 3-methylbenzoate as the sole C source (41), a property later shown to be associated with the presence of the TOL plasmid pWW0 (76). *P. putida* KT2440 is a cured, restriction-deficient derivative of *P. putida* mt-2 (13) which has been widely used in physiological and genetic studies (see references 48 and 51 for reviews). The nonpathogenic *P. putida* KT2440 has been shown to be an ideal host for expanding the range of substrates that it can degrade through the recruitment of genes from other microorganisms (52, 53). This strain has also been used as a vehicle for gene cloning and expression (36) and for the biotransformation of several chemicals in added-value products (8). This strain also colonizes the plant rhizosphere, which makes it potentially useful for phytorhizoremediation and for the development of biopesticides. These features make *P. putida* KT2440 a key strain within this genus (37, 49, 60). An international consortium is now considering sequencing its genome (73).

For efficient exploitation of this strain, thorough knowledge of its genome organization is essential. The development of pulsed-field gel electrophoresis (PFGE) and concomitant technology for the manipulation of large fragments of DNA (68) have revolutionized the analysis of bacterial chromosomes. More than 100 physical maps have been constructed (5, 12). For three members of the genus *Pseudomonas*—*Pseudomonas aeruginosa* PAO1, *Pseudomonas fluorescens* SBW25, and *Pseudomonas syringae* pv. phaseolicola—complete physical maps and detailed genetic maps have been constructed (7, 19, 46, 57, 65). In the case of *P. aeruginosa*, a sequencing project is providing in-depth knowledge about the species.

Unlike the situation for *P. aeruginosa*, little is known about the *P. putida* genome, and only an approximate chromosome map for one of the strains of this species has been generated, by conjugation and transduction analysis (38, 71). We present here a macrorestriction map of *P. putida* KT2440 developed from data obtained by PFGE and Southern blot analyses. The size of the circular chromosome was estimated to be 6.0 Mb, based on the size of the fragments generated from the digestion of the whole chromosome with I-*Ceu*I, *Pac*I, *Swa*I, and *Pme*I.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *P. putida* EEZ15, a phosphinothricin-resistant derivative of the prototroph *P. putida* KT2440, was described in an earlier publication (56). *P. putida* EEZ15K-1 through -34 are kanamycin-resistant (Km^r) mini-Tn*5* derivatives of *P. putida* EEZ15 (56). *Escherichia coli* JM109 was used to maintain different plasmids (66). Bacterial cells were grown at 30°C on Luria-Bertani (LB) culture medium. When necessary, ampicillin, chloramphenicol, kanamycin, and tetracycline were added to final concentrations of 100, 180, 25, and 15 μg/ml, respectively. Plasmids used for preparation of gene probes were extracted from *E. coli* host strains by the alkaline lysis method (66).

PFGE. (i) DNA preparation. Unsheared DNA was prepared by embedding whole cells in agarose blocks. *P. putida* cells were grown in LB to late exponential phase; to align the origins of chromosomal replication, the culture was supplemented with chloramphenicol (180 μg/ml) and maintained for another hour. Cells were harvested by centrifugation for 10 min at 1,400 × *g* and washed twice

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with PettIV buffer (10 mM Tris-HCl [pH 7.6], 1 M NaCl) (1). Cell suspensions of different cell densities were prepared in the same buffer and mixed with an equal volume of molten 1.6% (wt/vol) low-melting-point preparative-grade agarose (Bio-Rad) to obtain agarose plugs with 0.5×10^9, 1×10^9, and 2×10^9 cells/ml. Cells were lysed by submerging the plugs in EC-lysis solution (6 mM Tris-HCl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% [wt/vol] Brij 58, 0.2% [wt/vol] deoxycholate, 0.5% [wt/vol] sarcosyl, 1 mg of hen egg white lysozyme per ml, 20 μg of bovine pancreatic RNase per ml) for 24 h at 37°C. The EC-lysis solution was replaced with ESP solution (0.5 M EDTA [pH 9.5], 1% [wt/vol] lauryl sarcosine, 1 mg of proteinase K per ml), and incubation continued for a further 48 h at 50°C. The agarose blocks were washed several times with TE buffer (10 mM Tris-HCl, 10 mM EDTA [pH 8]) and stored at 4°C until use.

(ii) Restriction endonuclease digestion and end labeling. Endonucleases were purchased from New England Biolabs (*Pme*I, *Pac*I, and I-*Ceu*I) and Boehringer Mannheim (*Swa*I). Before restriction, one-third of an agarose block was equilibrated three times with 1 ml of the recommended restriction buffer, replaced with 60 μl of fresh restriction buffer supplemented with 20 μg of bovine serum albumin per ml, 7 mM dithiothreitol, and the appropriate amount of enzyme (4, 1, 2, and 5 U for *Pme*I, I-*Ceu*I, *Pac*I, and *Swa*I, respectively), incubated overnight at 4°C, and then incubated at 37°C for 2 h. Double digestion was done sequentially. End labeling of *Pme*I- or *Swa*I-digested DNA was achieved by incubating each plug with 5 μCi of [α-^{32}P]dTTP in 20 μl of Klenow buffer (10 mM $MgCl_2$, 50 mM NaCl, 10 mM Tris-HCl [pH 7.5]) with 1 U of Klenow enzyme for 30 min at room temperature. ^{32}P-labeled fragments were separated by conventional gel electrophoresis and transferred to nylon membranes as described below. ^{32}P-labeled fragments were detected by autoradiography.

(iii) CHEF electrophoresis. Contour-clamped homogeneous electric field (CHEF) electrophoresis was done in a Pharmacia-LKB Gene Navigator. Agarose gels (1.2% [wt/vol]) were run in 0.5× TBE buffer (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA [pH 8]) at 10°C unless otherwise stated. Voltage, pulse time, and total running time varied according to the size range of fragments to be separated. Specific conditions are provided in the legends for the corresponding figures. Lambda DNA concatemers (Pharmacia), laboratory-made lambda *Hin*dIII digest fragments, and chromosomes of *Saccharomyces cerevisiae* S-13 and *Hansenula wingei* (Bio-Rad) were used as molecular size DNA standards.

Southern hybridization and DNA labeling. DNA fragments separated in PFGE gels were irradiated with UV light (254 nm) for 2 min. DNA was transferred onto nylon membranes by capillary blotting for 48 h (70). Specific probes for hybridization were recovered from agarose gels with an agarose gel DNA extraction kit (Boehringer Mannheim). *P. putida* macrorestriction fragments used as DNA probes were obtained from a 1.2% (wt/vol) low-melting-point agarose PFGE gel by diluting the agarose with TE buffer to a final concentration of 0.3% (wt/vol) agarose, melting it at 68°C, and performing phenol, phenol-chloroform, and chloroform extractions. Finally, DNA was ethanol precipitated according to standard procedures (66). All probes were digoxigenin labeled by Klenow random primer extension according to the recommended procedure (DIG-DNA labeling and detection kit; Boehringer Mannheim). Blotted filters were prehybridized, hybridized, washed, and immunologically developed according to the supplier's instructions. High-stringency conditions (50% [vol/vol] formamide and 42°C) were used for *P. putida* gene probes. For heterologous gene probes, the conditions were less stringent: the concentration of formamide in the hybridization solution was decreased to 30% (vol/vol), or the hybridization temperature was reduced to 28°C. Blots were stored at −20°C and reused several times.

Amplification of DNA by PCR. Several probe templates were prepared by PCR amplification of genomic DNA. Genomic DNA from *P. putida* was prepared as described before (63). *P. putida* sequences were obtained from the EMBL database and used to design the following primers. For the cell division *ftsZ* gene, the primers 5′-GGCCCCAGTGCTTGAACGCT-3′ and 5′-TTAATCAGCCTG ACGACGCA-3′ were used; amplification yielded a 1.3-kb fragment. The gene *pyrB*, encoding aspartate carbamoyltransferase, was obtained after PCR amplification with the primers 5′-TACTGATGGGCGGTCGCACC-3′ and 5′-CCCG CTCATGGCCATGGACA-3′; a 1.2-kb fragment was obtained. The lipoamide dehydrogenase (*lpdG*) gene was obtained after PCR amplification with primers 5′-GCCAGGTCGTGATTCGCCCG-3′ and 5′-CCCGCCGTGGTTTCTTATA A-3′; it yielded a 1.7-kb fragment. To obtain the gene encoding muconate-lactonizing enzyme (*catB*), primers 5′-GACAAGCGCGCTGATTGAAC-3′ and 5′-ACAGCGACGGGCGAAGCGCG-3′ were chosen. All PCR amplifications were performed as recommended by the manufacturer in a Perkin-Elmer DNA thermal cycler under the following conditions: 1 min at 92°C, 1 min at 60°C, and 1 min at 72°C, except for *lpdG*, for which the annealing temperature was 45°C.

RESULTS

Choice of restriction enzymes, separation of fragments, and estimation of *P. putida* KT2440 genome size. To construct the physical map, the restriction enzymes chosen should be able to generate a manageable number of fragments. Our selection of appropriate rare-cutting endonucleases was based on the high G+C content (62.5 mol%) of *P. putida* (42). Three enzymes with rich A+T recognition sequences, *Dra*I, *Ssp*I, and *Xba*I, were assayed. These 6-bp recognition sequence enzymes generated more than 50 fragments each (data not shown) and were considered inappropriate. *Spe*I, which recognizes the rare tetranucleotide CTAG (35) within its 6-bp recognition sequence, was also tested. It produced more than 40 fragments, many less than 50 kb in size, and thus was not suitable for this study. *Pac*I, *Swa*I, and *Pme*I, which recognize an A+T-rich 8-bp target sequence, generated 6, 19, and 9 fragments respectively (Fig. 1; Table 1). These enzymes were considered suitable, as was I-*Ceu*I, which recognizes a specific 26-bp sequence within the gene encoding the 23S rRNA (30) and which cut the *P. putida* KT2440 genome in six fragments (Fig. 1; Table 1).

All DNA fragments generated by restriction were separated by CHEF electrophoresis. Different running conditions were required for optimal resolution in each size range (Fig. 1A through E). For example, a period of 9 days was needed to resolve the largest fragment detected in these analyses, i.e., the 2,850-kb I-*Ceu*I fragment A (Fig. 1A). Fragments in the size range between 1,000 and 65 kb were resolved under the running conditions specified in the legend to Fig. 1, so that the size of each fragment could be accurately determined (Fig. 1B through F). The smallest *Swa*I and *Pme*I single-digest fragments, which were smaller than 50 kb, could be detected by ethidium bromide staining (Fig. 1G). ^{32}P-end labeling of *P. putida* DNA digested previously with *Swa*I or *Pme*I did not resolve any additional fragments upon autoradiographic development (Fig. 2). Table 1 summarizes the sizes of the restriction fragments obtained with the endonucleases used in this work, averaged from more than 10 separate gels. The genome size of *P. putida* KT2440 was estimated by adding the sizes of the fragments generated by each of the endonucleases used; this yielded an average genome size of 6.0 Mb (Table 1) with an error of less than 2%.

The size of the genome was further confirmed when the sizes of the fragments resulting from double digestion with *Swa*I-*Pme*I and *Pme*I-*Pac*I (Table 2) were added. ^{32}P-end labeling of *P. putida* DNA digested with *Swa*I and *Pme*I revealed two additional fragments of 8 and 1 kb which were not detectable by ethidium bromide staining (Fig. 2).

In the course of mapping, the identity of the fragment patterns between successive plug preparations was checked. No change in fragment pattern was observed during the period of study.

Construction of the physical map. Two approaches were combined to organize the restriction fragments into a map: (i) physical methods and (ii) hybridization analysis.

(i) Physical methods. In addition to total digestion of the chromosomal DNA of *P. putida* with *Swa*I, *Pac*I, *Pme*I, and I-*Ceu*I, we tried to obtain partial digestions with these enzymes by reducing either the amount of enzyme or the incubation time. For I-*Ceu*I, two partial fragments (Fig. 1C) of 750 and 470 kb were observed. These fragments can correspond only to the combinations I-*Ceu*I-D (610 kb)–I-*Ceu*I-F (126 kb) and I-*Ceu*I-E (330 kb)–I-*Ceu*I-F (126 kb), respectively. Therefore, these results unequivocally link the fragments in the order I-*Ceu*I-D–I-*Ceu*I-F–I-*Ceu*I-E).

Partial digestion with *Swa*I generated a wide range of fragments, but only two of them (420 and 220 kb) could be unequivocally assigned to the combinations *Swa*I-H (352 kb)–*Swa*I-L (65 kb) and *Swa*I-J (180 kb)–*Swa*I-N (30 kb) (not shown).

To extend the limited information derived from partial digestion of chromosomal DNA, we analyzed the *Swa*I-*Pme*I double digests of the *P. putida* KT2440 chromosome. This was

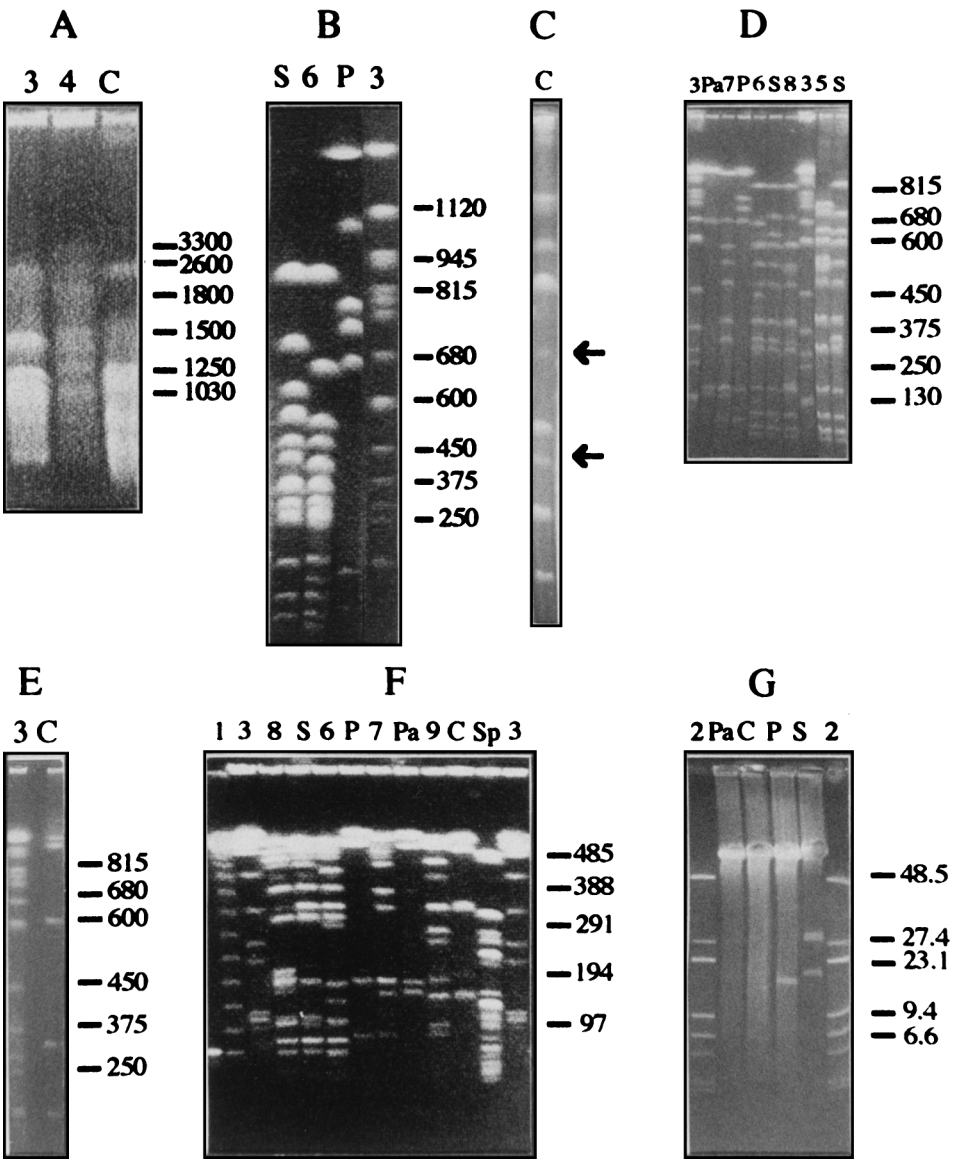

FIG. 1. CHEF electrophoresis of fragments of *P. putida* KT2440 genomic DNA predigested with different restriction enzymes. All gels were 1.2% (wt/vol) agarose and were run in 0.5× TBE buffer with the exception of gel A, which was a 0.8% (wt/vol) agarose gel run in 1× TBE buffer. (A) Running conditions: 50 V for 216 h; pulse times, 1,000 s for 24 h, followed by 1,000- to -4,000-s linearly ramped pulse for 192 h. Running conditions: (B and C) 150 V for 70 h; pulse times, 200 s for 24 h, 120 s for 24 h, and 80 s for 22 h. In gel C, partial I-*Ceu*I fragments are indicated by arrows. (D and E) Running conditions: 145 V for 66 h; pulse times, 120 s for 24 h, 70 s for 22 h, and 60 s for 20 h. (F) Running conditions: 100 V for 72 h; 5- to 100-s linearly ramped pulse. (G) Running conditions: 450 V for 4 h, pulse times, 0.5 s. Lanes: S, *Swa*I; P, *Pme*I; Pa, *Pac*I; C, I-*Ceu*I; Sp, *Spe*I; 6, SwaI plus PmeI; 7, *Pac*I plus *Pme*I; 8, *Swa*I plus *Pac*I; 9, I-*Ceu*I plus *Pme*I. DNA size markers were phage λ DNA concatemers (lane 1), intact phage λ DNA plus phage λ DNA digested with *Hin*dIII (lanes 2), and chromosomes of *S. cerevisiae* (lanes 3) and *H. wingei* (lanes 4). Lane 5 contains total *P. putida* DOT-OX3 DNA digested with *Swa*I. Sizes are indicated in kilobases.

expected to provide information about linkage of the fragments generated by *Swa*I and *Pme*I (Fig. 1B, D, and F). Fragment *Swa*I-A remained uncut by *Pme*I and was assumed to be contained within *Pme*I-A or *Pme*I-B. Fragment *Swa*I-B included a *Pme*I restriction site yielding a smaller fragment of 680 kb (Table 1). Fragments *Swa*I-C and *Swa*I-D were both cut by *Pme*I; one of them generated a 560-kb fragment (Fig. 1B), but the other fragment generated could not be identified unequivocally. *Swa*I-E and the double *Swa*I-G fragments, *Swa*I-H, *Swa*I-I, *Swa*I-K′, *Swa*I-L, and *Swa*I-M remained uncut by *Pme*I (Table 2; Fig. 1D and F).

To further exploit this information, we used a modified version of two-dimensional restriction fragment analysis as described by Bautsch et al. (2) and Römling et al. (65). Total chromosomal DNA was digested with *Pme*I, and the resulting fragments (*Pme*I-A through *Pme*I-G) were initially separated on a CHEF electrophoresis gel and identified (Fig. 3A). An agarose plug with each of the DNA bands was then removed from the gel and further digested with *Swa*I, and the new fragments were separated in a second electrophoresis (Fig. 3B). As a control, total chromosomal DNA of *P. putida* KT2440 was digested with *Swa*I. These analyses revealed that fragments *Swa*I-A, *Swa*I-H, *Swa*I-I, *Swa*I-L, *Swa*I-M, and *Swa*I-N were internal fragments of *Pme*I-A, and as expected, two new fragments were generated by *Swa*I within *Pme*I-A. These were called SP3 (450 kb) and SP12 (21 kb) (Table 2).

TABLE 1. Fragment sizes of all single restriction enzyme digests of *Swa*I, *Pme*I, I-*Ceu*I, and *Pac*I of the *P. putida* KT2440 chromosome

Fragment	Fragment size[a] (kb) obtained with:			
	*Swa*I	*Pme*I	I-*Ceu*I	*Pac*I
A	920	2,506	2,850	1,875
B	702	1,025	1,230	1,750
C	620	778	946	1,428
D	584	729	610	678
E	533	674	330	155
F	470	170	126	132
G	395[b]	78		
H	352	19		
I	325	18		
J	180			
K	115			
K′	105			
L	65[b]			
M	55			
N	30			
N′	29			
O	20			
Estimated total genome size (kb)	5,960	5,997	6,092	6,018

[a] Average of at least 10 independent gels. Standard deviations were in the range of 1 to 5% of the given value.
[b] The band is a doublet.

*Pme*I-B contained *Swa*I-E, *Swa*I-K′, and *Swa*I-G fragments and two new fragments, designated SP9 (60 kb) and SP13 (8 kb) (Table 2). *Pme*I-C generated fragments SP1 (680 kb) and SP8 (130 kb). *Pme*I-D contained the *Swa*I-N fragment and yielded two new fragments called SP2 (560 kb) and SP6 (172 kb). *Pme*I-E yielded SP4 (390 kb) and SP5 (330 kb) upon digestion with *Swa*I, whereas *Pme*I-F contained *Swa*I-O and yielded fragments SP7 (165 kb) and SP14 (1 kb). Digestion of *Pme*I-G with *Swa*I resulted in fragments SP10 (45 kb) and

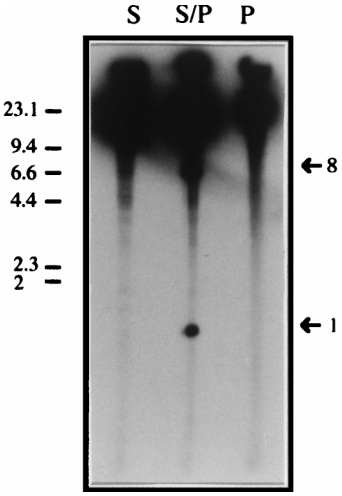

FIG. 2. Identification of genomic fragments of *P. putida* KT2440 smaller than 15 kb after digestion with *Swa*I, *Pme*I, and *Swa*I plus *Pme*I and ^{32}P end labeling. Total DNA of *P. putida* KT2440 genomic DNA was digested with *Swa*I (lane S), *Pme*I (lane P), and *Swa*I plus *Pme*I (lane S/P) and then end labeled with ^{32}P as described in Materials and Methods. Fragments were separated in a conventional 0.8% (wt/vol) agarose gel run in 1× TAE buffer for 3 h at 5 V cm^{-1}. The gel was exposed to Kodak photographic film and developed. Size fragments are indicated in kilobases. Bands of interest and their sizes are indicated by arrows.

TABLE 2. Fragment sizes resulting from double digestion of *P. putida* KT2440 DNA[a]

Fragment	Fragment size[b] (kb) obtained with *Swa*I + *Pme*I	Name	Fragment size (kb) obtained with *Pme*I + *Pac*I
*Swa*I-A	920	PP1	1,390
SP1	680	PP2	1,115
SP2	560	*Pme*I-E	678
*Swa*I-E	533	PP3	548
SP3	450	PP4	510
*Swa*I-G	395	PP5	446
SP4	390	PP6	384
*Swa*I-H	352	PP7	337
*Swa*I-I	325	*Pme*I-F	180
SP5	320	*Pac*I-E	155
SP6	172	*Pac*I-F	132
SP7	165	*Pac*I-G/PP8	78[c]
SP8	130	*Pme*I-H	19
*Swa*I-K′	105	*Pme*I-I	19
*Swa*I-L*	65[c]		
SP9	60		
*Swa*I-M	55		
SP10	45		
SP11/SwN	30*		
*Swa*I-N′	29		
SP12	21		
*Swa*I-O	20		
*Pme*I-H	18		
*Pme*I-I	18		
SP13	8		
SP14	1		
Estimated total genome size (kb)	5,977		6,068

[a] The fragments were designated *Swa*I, *Pme*I, or *Pac*I to indicate that the corresponding *Swa*I, *Pme*I, or *Pac*I fragment was not cut by *Swa*I, *Pme*I, or *Pac*I, respectively. The fragments resulting from the cut of a *Swa*I or *Pme*I fragment by the other enzyme are designated SP followed by a number (1 to 14) such that the higher the number, the smaller the DNA fragment. Accordingly, fragments were designated PP followed by a number when they resulted from the digestion of a *Pme*I fragment by *Pac*I and vice versa.
[b] Average of at least three independent gels. Standard deviations are in the range of 1 to 5% of the given values.
[c] The band is a doublet.

SP11 (30 kb). This approach allowed us to locate all of the *Swa*I *Pme*I-site-free fragments within a *Pme*I fragment. Based on these data, we linked some of the *Pme*I fragments to each other, on the assumption that the SP fragments generated in the *Swa*I/*Pme*I double digestion would equal the size of a previously determined *Swa*I fragment. For example, fragments SP3 (450 kb) and SP8 (130 kb) were assumed to come from *Swa*I-D (584 kb) and therefore to establish the linkage between *Pme*I-A and *Pme*I-C. Similar examples linked fragments *Pme*I-F with *Pme*I-E and *Pme*I-E with *Pme*I-B.

The order of the *Swa*I fragments included in *Pme*I-A could not be unequivocally assigned on the basis of the available information. Useful information was derived from strain DOT-OX3, a *P. putida* KT2440 mutant unable to synthesize the O antigen of the lipopolysaccharide that had been generated after mutagenesis with mini-Tn*5′ luxAB* (Fig. 1D, lane 4). When we tried to locate the position of the mini-Tn*5* on the chromosome of this strain, we found that fragments *Swa*I-A and *Swa*I-H had disappeared and two new fragments, of 750 and 480 kb, had appeared. The sum of the sizes of these two new fragments is similar to the sum of the sizes of *Swa*I-A and *Swa*I-H (Fig. 1D). This finding unequivocally linked these two

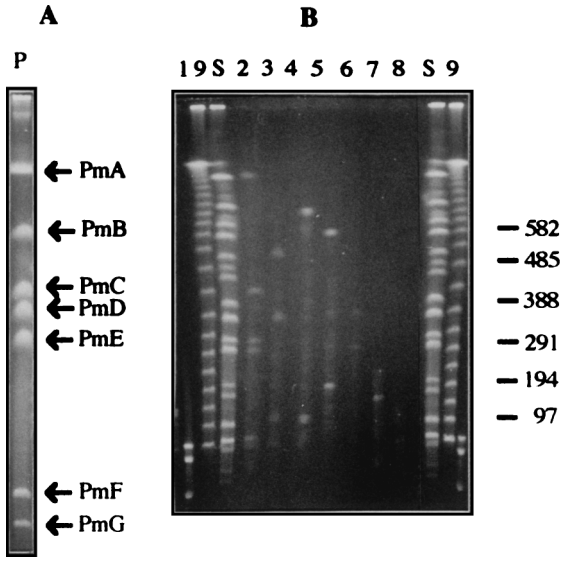

FIG. 3. Locations of *Swa*I restriction sites within *Pme*I fragments. (A) Separation of *P. putida* KT2440 *Pme*I fragments in the first electrophoresis; (B) separation of *Swa*I fragments generated upon digestion of different *Pme*I fragments. In gel A, two different lanes of total DNA digested with *Pme*I were run side by side. After ethidium bromide staining of one of them (A), the agarose plugs containing the *Pme*I fragment of interest were excised and digested with *Swa*I. Finally DNA fragments were separated in the second electrophoresis (B). Lanes: 1, phage lambda DNA undigested and digested with *Hin*dIII; 9, phage lambda DNA concatemers: S, *P. putida* KT2440 genomic DNA digested with *Swa*I. Lanes 2, 3, 4, 5, 6, 7, and 8 correspond to fragments *Pme*I-A, *Pme*I-B, *Pme*I-C, *Pme*I-D, *Pme*I-E, *Pme*I-F, and *Pme*I-G, respectively, digested with *Swa*I. Sizes are indicated in kilobases.

fragments and suggested that this mutant must contain an inversion of at least 150 kb.

In summary, the above series of analyses allowed us to establish that *Swa*I-A, *Swa*I-H, and *Swa*I-L were linked, as were *Swa*I-J and *Swa*I-N. We also found that fragments E, F, and D resulting from I-*Ceu*I digestion were also linked. *Pme*I-A was linked to *Pme*I-C, whereas *Pme*I-E was linked to *Pme*I-F and *Pme*I-B. The order of fragments in the intact chromosome was *Pme*I-F,E,B. We identified a number of *Swa*I fragments within each of the *Pme*I fragments. In all, the above pattern of fragment linkage provided a low-resolution map in which approximately two-thirds of the chromosome backbones have been established.

(ii) Hybridization analysis. To enhance the physical map, we used Southern blot analysis of a series of *P. putida* KT2440 derivatives labeled with mini-Tn*5*-Km. This series of mutants is called *P. putida* EEZ15K-*x* where *x* is 3, 8, 12, 14, 16, 17, 22, 23, 24, or 30 (56). As a probe, the Km^r determinant gene was used and the position of the Km^r gene in *P. putida* EEZ15K-*x* was established. Table 3 summarizes the hybridization data obtained for the DNA of each mutant cut with *Swa*I, *Pme*I, and I-*Ceu*I. This analysis confirmed the location of the different *Swa*I fragments within the *Pme*I fragments. In addition, it revealed that fragments I-*Ceu*I-E, I-*Ceu*I-F, and I-*Ceu*I-D were located within *Pme*I-A. We also found that I-*Ceu*I-C was contained within *Pme*I-A, as surmised from evidence that the Km^r cassette in *P. putida* EEZ15K-30 was located in *Swa*I-A, *Pme*I-A, and I-*Ceu*I-C. The same was true for EEZ15K-16 except that in this mutant the mini-Tn*5* lies within the *Swa*I-H fragment. The finding that the Km^r cassette in mutant EEZ15K-23 was located in *Swa*I-L and I-*Ceu*I-E and that the mini-Tn*5* in mutant EEZ15K-14 was in *Swa*I-I and in I-*Ceu*I-E

TABLE 3. Localization of the Km^r determinant in the chromosomes of Km^r derivatives of *P. putida* EEZ15K-*x*[a]

Km^r mutant	Fragment		
	*Swa*I	*Pme*I	I-*Ceu*I
EEZ15K-3	I	A	F
EEZ15K-8	K′	B	A
EEZ15K-12	D	A	D
EEZ15K-14	I	A	E
EEZ15K-16	H	A	C
EEZ15K-17	C	D	A
EEZ15K-22	B	C	B
EEZ15K-23	L	A	E
EEZ15K-24	G	E	A
EEZ15K-30	A	A	C

[a] Conditions for digestion and separation of fragments were as described in the legend for Fig. 1A, B, and D.

linked *Swa*I-I with the set of fragments *Swa*I-A–*Swa*I-H–*Swa*I-L.

Given that *Swa*I-D established the linkage between *Pme*I-A and *Pme*I-C, the position of the Km^r cassette in mutant EEZ15K-22 within fragments *Swa*I-B, *Pme*I-C, and I-*Ceu*I-B confirmed the connection between fragments *Swa*I-D and *Swa*I-B as well as that between fragments I-*Ceu*I-D and I-*Ceu*I-B.

We deduced that the largest I-*Ceu*I-A fragment included *Pme*I-B, *Pme*I-D, and *Pme*I-E, because mutants EEZ15K-8, EEZ15K-17, and EEZ15K-24 were located within these fragments (Table 3). From the information thus obtained, the linkage of all I-*Ceu*I fragments was established as I-*Ceu*I-A,C,E,F,D,B.

In addition, the 51 gene probes listed in Table 4 were used against *P. putida* KT2440 chromosomal DNA digested with *Swa*I, *Pme*I, I-*Ceu*I, *Pac*I, *Swa*I-*Pme*I, etc. (Tables 5 and 6). This allowed us to further define the map. As an example, the hybridization gel with the *npt* gene probe and the *dnaJ-dnaB-carAB* set of genes is shown in Fig. 4.

Analysis of all hybridization assays unequivocally located all I-*Ceu*I and *Pac*I fragments except fragments *Pac*I-E and *Pac*I-F, which, because they showed the same location within *Swa*I-C, *Pme*I-D, and I-*Ceu*I-A, could be exchanged with each other. We were able to locate most but not all *Swa*I and *Pme*I fragments. The positions of the smaller *Swa*I (*Swa*I-K through -O) and *Pme*I (PmeI-G through -I) fragments remained undetermined. These fragments were separated with PFGE, and fragments smaller than 130 kb were extracted and purified from agarose gels (Table 6). These fragments were used directly as probes against blots of chromosomal DNA digested with *Swa*I, *Pme*I, and I-*Ceu*I. A single *Swa*I or *Pme*I fragment used as a probe should produce a single band when hybridized against a filter carrying fragments of a digest produced by the same enzyme and should produce one or more bands when hybridized to a digest produced by one of the other enzymes. The data from these experiments are summarized in Table 7. Figure 5 shows an example in which the *Swa*I-J fragment was used as a probe. It hybridized with itself (180 kb) and with the *Pme*I-D fragment (729 kb) in single-digest assays and with the SP6 (172 kb) fragment in the *Pme*I-*Swa*I double digest (Fig. 5). It also hybridized with the I-*Ceu*I fragment A (not shown). Similar assays allowed us to unequivocally locate the *Swa*I-G, *Swa*I-J, *Swa*I-K′, and *Swa*I-O fragments, as well as the *Pme*I-F, *Pme*I-G, *Pme*I-H, and *Pme*I-I fragments (Table 7).

Hybridization with fragments *Swa*I-L and *Swa*I-M produced multiple bands with all of the enzymes tested; these bands

TABLE 4. Gene probes used for hybridization in this study

Gene	Function	Species of origin	Reference(s)
arg	Arginine requirement	*Pseudomonas fluorescens*	46
bkdR	Positive transcriptional activator of the *bkd* operon	*P. putida*	31
catB	Muconate-lactonizing enzyme	*P. putida*	21
dnaJ, *dapB, carA,B*		*P. fluorescens*	45
dctA	C_4-dicarboxylate transport	*P. fluorescens*	4
fabF	Fatty acid synthesis	*Escherichia coli*	32
fliA, *cheY*	Flagellin synthesis, chemotaxis factors	*P. aeruginosa*	34
fliP,R	Flagellar biosynthetic genes	*P. putida*	69
ftsZ	Cell division protein	*P. putida*	79
gltB	Major subunit of glutamate dehydrogenase	*P. putida*	10
gyrB	DNA gyrase subunit B	*P. putida*	77
hemA, *prf1M*	Glutamyl-tRNA reductase, protein release factor 1	*P. aeruginosa*	24
hemB	5-Aminolevulonic acid dehydratase	*P. aeruginosa*	23
hemL	Glutamate-1-semialdehyde-1,2-aminomutase	*P. aeruginosa*	23
hemN	Oxygen-independent copro-porphyrinogen III-dehydrogenase	*P. aeruginosa*	23
qor, *hemF*, *aroE*	Quinone oxidoreductase, Oxygen-dependent copro-porphyrinogen III-oxidase, shikimate 5-dehydrogenase	*P. aeruginosa*	72
IS*1246*	Insertion sequence	*P. putida*	59
hip	Integration host factor subunit	*P. putida*	33
him	Integration host factor subunit	*P. putida*	33
leu	Leucine requirement	*P. fluorescens*	46
lexA	Sodium dodecyl sulfate	*P. putida*	3
lpdG	Lipoamide dehydrogenase	*P. putida*	43
lps	Lipopolysaccharide biosynthesis	*P. putida*	54
modABCD	Molybdate transport operon	*E. coli*	58
msrA	Methionine sulfoxide reductase	*E. coli*	39
npt	Assimilatory nitrate reductase gene	*P. putida*	10
oriC	Chromosomal replication origin	*P. aeruginosa*	78
osmE	Osmotically inducible envelope protein	*E. coli*	15
tolQRAB, *orpL*, *orf2*	Peptidoglycan-associated lipoprotein, cell envelope Tol complex periplasmic proteins	*P. putida*	64
panB,C	α-Ketopantoate hydroxymethyl transferase, pantothenate synthetase	*P. fluorescens*	46
pca, *qui*, *pob*	Protocatechuate *p*-hydroxybenzoate catabolism	*Acinetobacter calcoaceticus*	11
pcaG,H	Protocatechuate metabolism	*P. putida*	40
pcaK,F	4-Hydroxybenzoate transport protein, β-ketoadipyl coenzyme A thiolase	*P. putida*	17
pilK	Pilin biosynthesis	*E. coli*	6
putC	Regulator region of the proline operon	*P. putida*	47
pyrB	Aspartate carbamoyltransferase	*P. putida*	67
recA	RecA protein	*P. aeruginosa*	20
rpoD	RpoD σ factor	*P. putida*	16
rpoE	RpoE σ factor	*E. coli*	18
rpoN	RpoN σ factor	*P. putida*	26
rpoS	RpoS σ factor	*P. putida*	55
rrnA–F	rRNA operons	*P. aeruginosa*	27
thr	Threonine requirement	*P. fluorescens*	46
thrS, *infC rpmL, rplT*		*P. syringae*	25
trkA	Putative potassium uptake system	*P. putida*	69
ttgA,B, oprD	Toluene-tolerant genes	*P. putida*	50, 69
uvrA	UV resistance	*P. aeruginosa*	62
uvrB	UV resistance	*P. aeruginosa*	61

represent *rrn* operons within these fragments, as shown by hybridization with the *rrn* gene probe (Fig. 6). Fragments *Swa*I-L and *Swa*I-M were located on the basis of the positions of the I-*Ceu*I restriction sites and the hybridization data.

Construction of the gene map. Forty-five previously identified genes or gene clusters were located on the *P. putida* KT2440 physical map (Tables 5 to 7; Fig. 7) by probing chromosomal digests with available cloned genes and PCR fragments. The gene probes used for this purpose are listed in Table 4.

The backbone of the chromosome is defined by the position of the *rrn* loci *rrs* (16S), *rrl* (23S), and *rrf* (5S) and their distribution with respect to the origin of replication (5). I-*Ceu*I recognizes a specific 26-bp sequence within the *rrl* gene. The mapped I-*Ceu*I restriction sites localized six *rrn* operons, which we have designated *rrnA* through *-F*, in the physical map of *P. putida* KT2440 (Fig. 7). A specific *rrs* probe was used to validate the existence of an *rrn* operon within the *Swa*I and *Pme*I fragments that contain the I-*Ceu*I restriction sites (Fig. 6). Hybridization of the *rrs* probe to I-*Ceu*I chromosomal digests gave no signal with fragment I-*Ceu*I-B but gave a signal with the rest of the I-*Ceu*I fragments (Fig. 6).

Probes consisting of more than a single gene, such as *hemA-hemM-prf1*, *dnaJ-dapB-carAB*, and *pcaG-pcaH*, hybridized to a

TABLE 5. Fragments restricted with *Swa*I, *Pme*I I-*Ceu*I, and *Pac*I which gave a hybridization signal with several gene probes

Probe(s)	Restriction fragments hybridizing to[a]:			
	*Swa*I	*Pme*I	I-*Ceu*I	*Pac*I
arg	H (H + L)	A	C	
catB	C	D		
dnaJ, *dapB*, *carA*,*B*	A	A		
dctA	B			
fliA, *cheYZ*	J	D	A	B
fliP,*R*	J	D		
ftsZ	B	C	B	
gltB	A	A		
gyrB	H (H + L)	A	C	C
hemA,*M*, *pRf1*	D	A	D	C
hemL	A	A	C	B
hemN	J (J + N)	D	A	
qor, *hemF*, *aroE*	H	A	C	
IS*1246*	G	B	A	A
hip	B	C	B	
him	G	E	A	
leu	A	A	C	
lexA	F	E	B	
lpdG	D	A		
lps	B	C		A
npt	B	C		
oriC	H	A	C	
pal, *tolB*	B	C	B	
panB	A	A	C	
pcaG	A	A	C	
pcaK	B	C	B	
pilK	Unspecific signal			
putC	A			
pyrB	A		C	
recA	B	C	B	A
rpoD	I	A	E	C
rpoN	D	A	D	C
rpoS	B	C	B	A
rrs	A, D, F, I, L, M	A, C, E		
thr	B	C	B	
thrS, *infC*, *rpmL*, *rplT*	G	E	A	
trkA	A	A		
ttgA,*B*, *oprD*, *rpmL* *rplT*	B	C		
uvrA	I	A	F	
uvrB	F	F	B	

[a] Fragments in parentheses indicate that a hybridization signal with the indicated partial digestion fragment was obtained.

single band only, suggesting that these genes are probably contiguous in *P. putida* KT2440. The positions of the genes necessary for leucine, threonine, and arginine biosynthesis were mapped by Southern hybridization with cosmid clones from a *P. fluorescens* SBW25 genomic library (46). Each clone hybridized to a single band under high-stringency conditions, showing that these species are similar in gene organization within these cosmid areas.

No homology was detected to several *E. coli* probes (*fabF*, *asmE*, *mobABCD*, *msrA*, and *rpoE*) or to *pca-qui-pob* from *Acinetobacter calcoaceticus*, to *bkdR* from *P. putida* PpG2, or to *hemB* from *P. aeruginosa*, even under low-stringency conditions.

DISCUSSION

We have generated the first complete physical map for the enzymes I-*Ceu*I, *Pac*I, *Pme*I, and *Swa*I in *P. putida* KT2440. A single chromosome was shown to be circular and to have an estimated size of 6.0 Mb. Two independent mapping approaches, (i) analysis of fragments resulting from digestion of the whole genome with rare-cutting restriction enzymes and (ii) Southern hybridization, were used to minimize possible errors and to validate the data obtained separately with each approach. A similar method was used to construct the maps of *Haemophilus influenzae* (29) and *Mycoplasma mycoides* (44). In the *P. putida* KT2440 map, a total of 40 restriction sites (6 I-*Ceu*I, 6 *Pac*I, 9 *Pme*I, and 19 *Swa*I) were positioned on the map, achieving an average resolution of approximately 160 kb. Our procedure ensures that DNA fragments larger than 18 kb in size were detected by ethidium bromide staining of whole genome DNA and that the use of ^{32}P-labeled fragments detected fragments of up to 1 kb. It is unlikely that we overlooked fragments smaller than 1 kb because we avoided overrunning the gels when fragments were labeled with ^{32}P. The genome of this *P. putida* strain is only 100 kb larger than the 5.9-Mb *P. aeruginosa* PAO1 genome (65), 1.78 Mb larger than the average *Pseudomonas stutzeri* genome (14), about 400 kb larger than the *P. syringae* pv. phaseolica genome (7), and about 600 kb smaller than the 6.63-Mb *P. fluorescens* SWB25 genome (46).

A partial genetic map of the *P. putida* KT2440 genome was obtained by Southern hybridization with 51 probes (Table 4), of which 38 gave positive signals locating a total of 63 genes (Fig. 7), including key markers such as *oriC*, *recA*, *gyrB*, *rpoS*, *rpoN*, *rpoD*, and the rDNA operons. Figure 7 summarizes the results obtained for more than 100 gels resulting from single or double digestion with rare-cutting enzymes in a large series of hybridization experiments. Several auxotrophic markers (*leu*, *thr*, and *arg*) were positioned, as were genes involved in pilus biosynthesis and motility, lipopolysaccharide production, and inorganic nitrogen assimilation. In addition, we located several catabolic operons (genes) for the metabolism of aromatic compounds. *P. putida* KT2440 is a nonaggressive root colonizer

TABLE 6. Fragments restricted with *Swa*I/I-*Ceu*I, *Swa*I/*Pme*I, I-*Ceu*I/*Pme*I, *Pac*I/*Pme*I, and *Pac*I/*Swa*I which gave a hybridization signal with several gene probes

Probe	Restriction fragments hybridizing to[a]:				
	*Swa*I/ I-*Ceu*I	*Swa*I/ *Pme*I	I-*Ceu*I/ *Pme*I	PacI/ *Pme*I	*Pac*I/ *Swa*I
arg	*Swa*I-H	*Swa*I-H	*Ceu*I-C		
catB		SP2			
gyrB		*Swa*I-H	*Ceu*I-C		
him	*Swa*I-G	*Swa*I-G	420 kb		
leu		*Swa*I-A	*Ceu*I-C		
lexA		SP5	320 kb		
lpdG		SP3			
lps				PP5	475 kb
npt		SP1			
panB,*C*		*Swa*I-A	*Ceu*I-C		
putC	640 kb				
recA		SP1		PP5	475 kb
rpoD		*Swa*I-I	*Ceu*I-E		*Swa*I-I
rpoN	*Swa*I-D	SP3	494 kb		
rpoS		SP1	739 kb		475 kb
thr	700 kb	SP1	660 kb		
thrS, *infC*, *rpmL*		*Swa*I-G	420 kb		
trkA		*Swa*I-A			
urvB		SP7	*Pme*I-F		

[a] The fragment to which the probe hybridized is shown. The fragment is designated by a name when it was unequivocally identified in single or double digests of the whole genome; otherwise it is designated by fragment size.

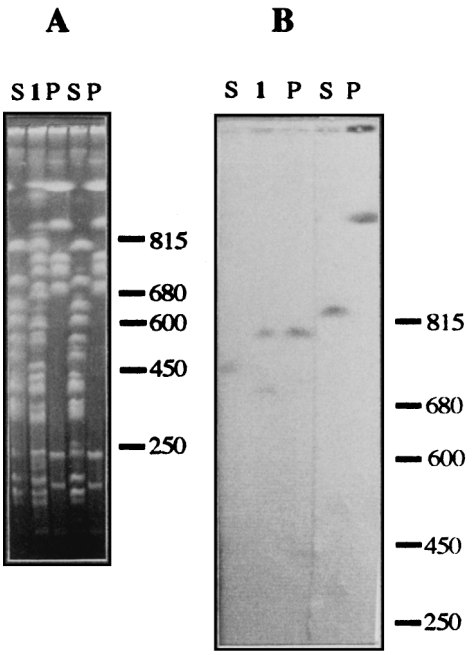

FIG. 4. Hybridization analysis with several gene probes. *P. putida* KT2440 DNA was digested with *Swa*I (lanes S), *Pme*I (lanes P), and *Swa*I plus *Pme*I (lanes 1), and DNA fragments were separated by CHEF electrophoresis. The three lanes on the left were separated from the two other lanes, and each set of DNA was transferred to a nylon membrane. In panel B, the three lanes on the left correspond to hybridization with the *npt* gene probe, while the other two lanes correspond to hybridization with the *dnaJ-dapB-carAB* set of genes. Sizes are indicated in kilobases.

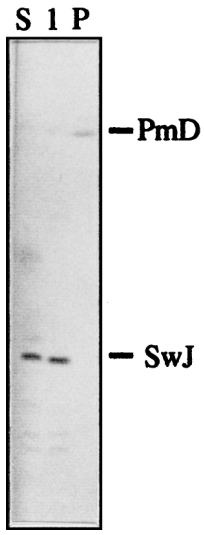

FIG. 5. Hybridization analysis with small chromosomal fragments. Fragment *Swa*I-J was extracted from a gel and used as a probe against *P. putida* KT2440 DNA digested with *Swa*I (lane S), *Pme*I (lane P), or *Swa*I plus *Pme*I (lane 1).

(37). The genes involved in C_4-dicarboxylate transport (*dctA*, *-B*, and *-D*) are important for rhizosphere colonization (37). In this study, we located the *dctA* gene.

The distribution of signals allowed us to identify a genetically dense region around *oriC*; the *rrn* operons seem to be grouped in this region with five of the six operons occupying one-third of the genetic map (Fig. 7). *P. putida* contains six rRNA loci, designated *rrnA* through *-F*. Localization of the I-*Ceu*I sites precisely positioned the *rrn* operons (30). Assuming the 5′-16S-23S-3′ orientation, the hybridization data with a 16S rRNA probe suggested that the rDNA genes are organized in a typical eubacterial manner, with the *rrn* operons transcribed divergently. The six copies of *rrn* operons in *P. putida* KT2440 contrast with the five *rrn* operons in *P. fluorescens* SBW25 (46) and *P. syringae* pv. phaseolica (7) and the four described for *P. aeruginosa* PAO1 (65). Among eubacteria, the number of rRNA operons varies between 14 in *Clostridium*

TABLE 7. Hybridization with genomic restriction fragments isolated from agarose gels

Probe	Restriction fragment(s) hybridizing to[a]:			
	*Swa*I	*Swa*I + *Pme*I	*Pme*I	I-*Ceu*I
*Pme*I-F	F	ND	F	B
*Pme*I-G	C	ND	G	ND
*Pme*I-H	C	ND	H	ND
*Pme*I-I	B	ND	I	ND
*Swa*I-G	G	G	A/E	ND
*Swa*I-J	J	SP6	D	A
*Swa*I-K	K (L) (M)	SP10	G	ND
*Swa*I-K′	K′	K′	B	ND
*Swa*I-L	MB	ND	MB	MB
*Swa*I-M	MB	ND	MB	MB
*Swa*I-N,N′	N, N′ (A)	ND	A/D (B) (C)	ND
*Swa*I-O	O	ND	F	B

[a] Fragments in parentheses gave weaker hybridization signals and were not necessarily adjacent. These weak signals were thought to arise from the presence of repeated sequences contained in the genomic restriction fragments. ND, not determined; MB, multiple bands.

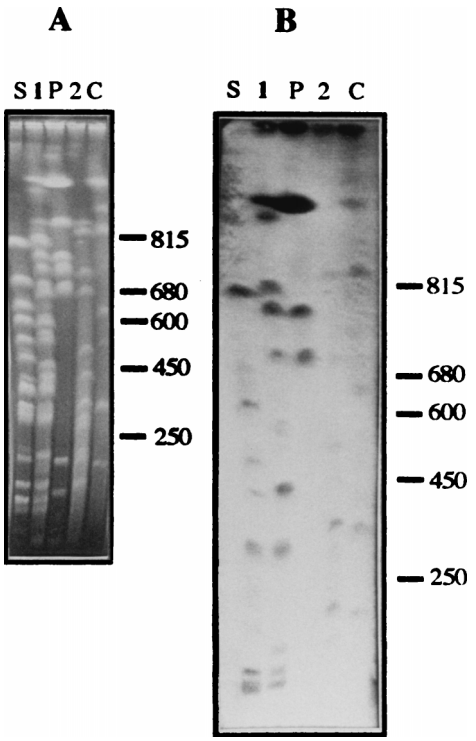

FIG. 6. Hybridization analysis with the *rrs* gene as a probe. *P. putida* KT2440 genomic DNA was digested with *Swa*I (lanes S), *Pme*I (lanes P), I-*Ceu*I (lanes C), *Swa*I plus *Pme*I (lanes 1), or I-*Ceu*I plus *Pme*I (lanes 2). The sizes (in kilobases) indicated on the right correspond to *S. cerevisiae* chromosomes used as a size marker.

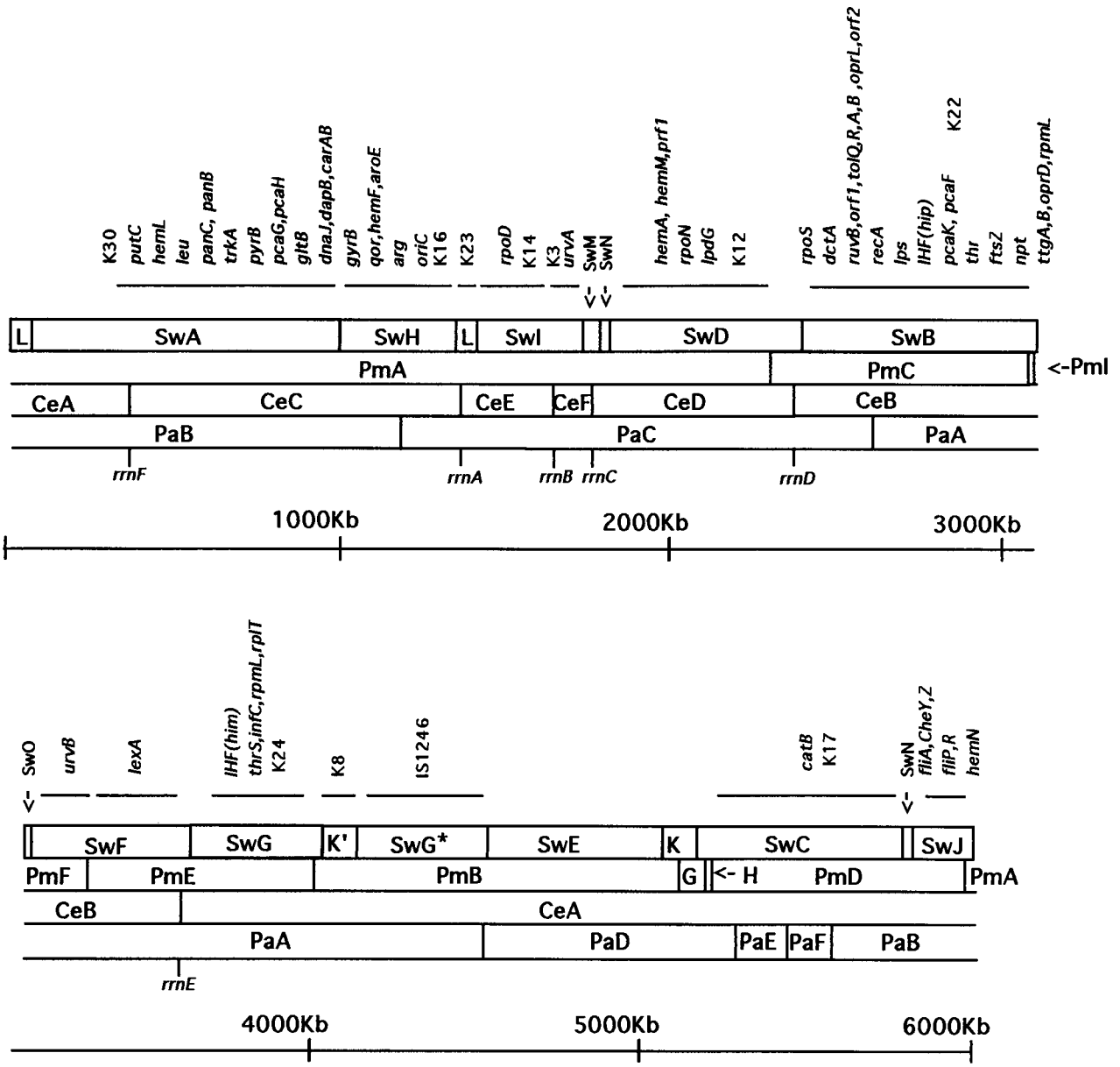

FIG. 7. Physical and genetic map of *P. putida* KT2440. The circular chromosome is represented as a series of overlapping fragments for the enzymes *Swa*I (Sw), *Pme*I (Pm), *Pac* (Pa), and I-*Ceu*I (Ce). Genetic loci were assigned to restriction fragments by Southern hybridization analysis; groups of markers that hybridize at the same restriction fragments are underlined. The exact positions of the six rDNA operons are indicated.

beijerinkii (75) and 1 in some species of the genus *Mycobacterium* (28). This finding suggests different patterns of gene rearrangements not only among different genera but also within the same genus.

This information provides a basis for comparing different members of the genus *Pseudomonas*. Figure 8 shows the locations of the common genetic markers in *P. putida* KT2440, *P. fluorescens* SBW25, and *P. aeruginosa* PAO1. It is accepted that translocatable elements allow bacterial chromosomes to acquire new genes by lateral transfer from other species, so that a different map location for genes performing the same function in species derived from a common ancestor does not necessarily reflect a chromosomal rearrangement since the time of divergence, but may mean that the two species have independently acquired that gene (27). However, the presumed ancestor of *P. aeruginosa*, *P. putida*, and *P. fluorescens* probably already possessed all or many of the genes required for DNA recombination, metabolism of simple metabolites, and uptake of compounds abundant in the environment (such as dicarboxylic acids), and so these markers should be reliable indicators of chromosomal rearrangements. The limited number of hybridization experiments with cosmids derived from *P. fluorescens* suggests that at least in the cases we studied, clusters of genes were conserved, although our limited information does not allow us to draw definitive conclusions. A detailed comparison of the genomic organization of different pseudomonads thus awaits further experimental analysis.

Determination of the physical and partial genetic map of the

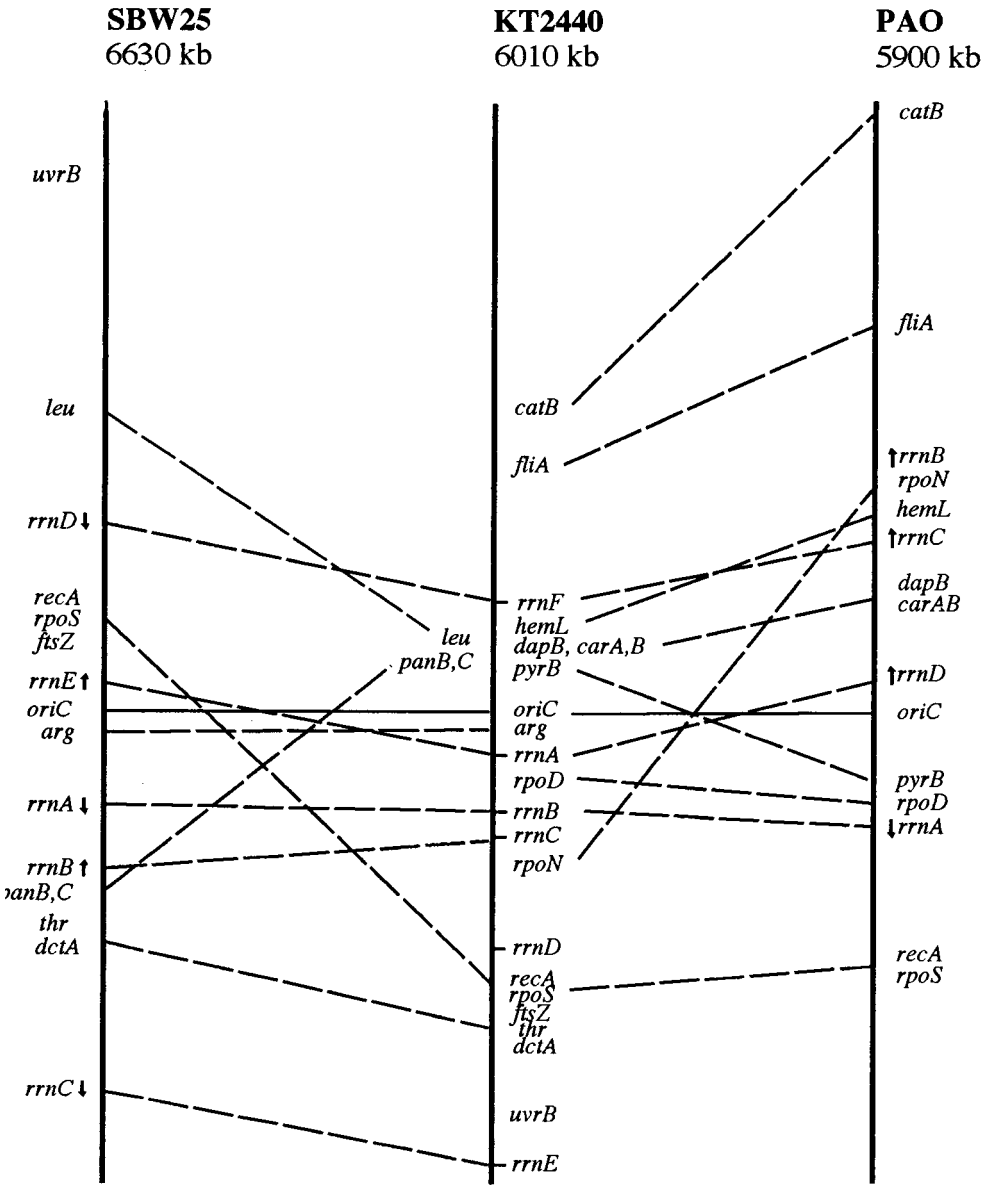

FIG. 8. Comparison of the maps of *P. fluorescens* SBW25, *P. putida* KT2440, and *P. aeruginosa* PAO1. To facilitate comparisons, the circular maps were opened at an arbitrarily chosen point and positioned with respect to the origin of replication. Only the genes found in both *P. putida* and one of the other bacteria are shown, connected by a dashed line. The position of each gene is expressed as a ratio of the position of that gene relative to *oriC*.

P. putida genome constitutes a significant step forward in terms of comparative genome analysis and will aid the genome sequencing project. The map provides a sound framework for studies of the taxonomy of *Pseudomonas* species as well as for studies of the colonization of different niches and the utilization of different nutrients by these bacteria.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1991. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
2. **Bautsch, W., D. Grothues, and B. Tümmler.** 1988. Genome fingerprinting of *Pseudomonas aeruginosa* by two dimensional field inversion gel electrophoresis. FEMS Microbiol. Lett. **52:**255–258.
3. **Calero, S., X. Garriga, and J. Barbé.** 1993. Analysis of the DNA damage-mediated induction of *Pseudomonas putida* and *Pseudomonas aeruginosa lexA* gene. FEMS Microbiol. Lett. **110:**65–70.
4. **Challis, B. C.** 1994. Molecular characterization of the C4-dicarboxylate transport system of *P. fluorescens* and its role in root colonization. M.Sc. thesis. University of Otago, Dunedin, New Zealand.

5. **Cole, S. T., and I. Saint Girons.** 1994. Bacterial genomics. FEMS Microbiol. Rev. **14:**139–160.
6. **Darzins, A.** 1993. The *pilG* gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single-domain response regulator CheY. J. Bacteriol. **175:**5934–5944.
7. **de Ita, M. E., R. Marsch-Moreno, P. Guzmán, and A. Alvarez-Morales.** 1998. Physical map of the chromosome of the phytopathogenic bacterium *Pseudomonas syringae* pv. *phaseolica*. Microbiology **144:**493–501.
8. **Delgado, A., M. G. Wubbolts, M. A. Abril, and J. L. Ramos.** 1992. Nitroaromatics are substrates for the TOL plasmid upper-pathway enzymes. Appl. Environ. Microbiol. **58:**415–417.
9. **de Weger, L. A., P. A. H. M. Bakker, B. Schippers, M. C. M. van Loosdrecht, and B. J. J. Lugtenberg.** 1989. *Pseudomonas* spp. with mutational changes in the O-antigenic side chain of their lipopolysaccharide are affected in their ability to colonize potato roots. NATO ASI (Adv. Sci. Inst.) Ser. **H36:**197–202.
10. **Eberl, L.** 1998. Personal communication.
11. **Elsemore, D. A., and L. N. Ornston.** 1994. The *pca-pob* supraoperonic cluster of *Acinetobacter calcoaceticus* contains *quiA*, the structural gene for quinate-shikimate dehydrogenase. J. Bacteriol. **176:**7659–7666.
12. **Fonstein, M., and R. Haselkorn.** 1995. Physical mapping of bacterial genomes. J. Bacteriol. **177:**3361–3369.
13. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta*-cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
14. **Ginard, M., J. Lalucat, B. Tummler, and U. Römling.** 1997. Genome organization of *Pseudomonas stutzeri* and resulting taxonomic and evolutionary considerations. Int. J. Syst. Bacteriol. **47:**132–143.
15. **Gutierrez, C., S. Gordia, and S. Bonnassie.** 1995. Characterization of the osmotically inducible gene *osmE* of *Escherichia coli* K-12. Mol. Microbiol. **16:**553–563.
16. **Harayama, S.** Personal communication.
17. **Harwood, C. S., N. N. Nichols, M. K. Kim, J. L. Ditty, and R. E. Parales.** 1994. Identification of the *pcaRKF* gene cluster from *Pseudomonas putida*: involved in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. J. Bacteriol. **176:**6479–6488.
18. **Hiratsu, K., M. Amemura, H. Nashimoto, H. Shinagawa, and K. Makino.** 1995. The *rpoE* gene of *Escherichia coli*, which encodes σ^E, is essential for bacterial growth at high temperature. J. Bacteriol. **177:**2918–2922.
19. **Holloway, B. W., U. Römling, and B. Tümmler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiology **140:**2907–2929.
20. **Horn, J. M., and D. E. Ohman.** 1988. Transcriptional and translational analyses of *recA* mutant alleles in *Pseudomonas aeruginosa*. J. Bacteriol. **170:**1637–1650.
21. **Houghton, J. E., T. M. Brown, A. J. Appel, E. J. Hughes, and L. N. Ornston.** 1995. Discontinuities in the evolution of *Pseudomonas putida cat* genes. J. Bacteriol. **177:**401–412.
22. **Housiaux, P. J., D. F. Hill, and G. B. Peterson.** 1988. Nucleotide sequence of a gene for 5S ribosomal RNA from *Pseudomonas aeruginosa*. Nucleic Acids Res. **16:**2721.
23. **Hungerer, C., B. Troup, U. Römling, and D. Jahn.** 1995. Cloning, mapping and characterization of the *Pseudomonas aeruginosa hemL* gene. Mol. Gen. Genet. **248:**375–380.
24. **Hungerer, C., D. S. Weiss, R. K. Thauer, and D. Jahn.** 1996. The *hemA* gene encoding glutamyl-tRNA reductase from the archaeon *Methanobacterium thermoautotrophicum* strain Marburg. Bioorg. Med. Chem. **4:**1089–1095.
25. **Kitten, T., and D. K. Willis.** 1996. Suppression of a sensor kinase-dependent phenotype in *Pseudomonas syringae* by ribosomal proteins L35 and L20. J. Bacteriol. **178:**1548–1555.
26. **Köhler, T., S. Harayama, J. L. Ramos, and K. N. Timmis.** 1989. Involvement of *Pseudomonas putida* RpoN σ factor in regulation of various metabolic functions. J. Bacteriol. **171:**4326–4333.
27. **Kolstø, A.-B.** 1997. Dynamic bacterial genome organization. Mol. Microbiol. **24:**241–248.
28. **Krawiec, S., and M. Riley.** 1990. Organization of the bacterial chromosome. Microbiol. Rev. **54:**502–539.
29. **Lee, J. J., H. O. Smith, and R. J. Redfield.** 1989. Organization of the *Haemophilus influenzae* Rd genome. J. Bacteriol. **171:**3016–3024.
30. **Liu, S., A. Hessel, and K. E. Sanderson.** 1993. Genomic mapping with I-*Ceu*I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. Proc. Natl. Acad. Sci. USA **90:**6874–6878.
31. **Madhusudhan, K. T., N. Huang, and J. R. Sokatch.** 1995. Characterization of BkdR-DNA binding in the expression of the *bkd* operon of *Pseudomonas putida*. J. Bacteriol. **177:**636–641.
32. **Magnuson, K., M. R. Carey, and J. E. Cronan, Jr.** 1995. The putative *fabJ* gene of *Escherichia coli* fatty acid synthesis is the *fabF* gene. J. Bacteriol. **177:**3593–3595.
33. **Marqués, S., M. T. Gallegos, M. Manzanera, A. Holtel, K. N. Timmis, and J. L. Ramos.** 1998. Activation and repression of transcription at the double tandem divergent promoters for *xylR* and *xylS* genes of the TOL plasmid of *Pseudomonas putida*. J. Bacteriol. **180:**2889–2894.
34. **Masduki, A., J. Nakamura, T. Ohga, R. Umezaki, J. Kato, and H. Ohtake.** 1995. Isolation and characterization of chemotaxis mutants and genes of *Pseudomonas aeruginosa*. J. Bacteriol. **177:**948–952.
35. **McClelland, M., R. Jones, Y. Patel, and M. Nelson.** 1987. Restriction endonucleases for pulsed-field mapping of bacterial genomes. Nucleic Acids Res. **15:**5985–6005.
36. **Mermod, N., P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1984. Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated overlapping promoters. EMBO J. **11:**2461–2466.
37. **Molina, L., C. Ramos, M. C. Ronchel, S. Molin, and J. L. Ramos.** 1998. Field release of biologically contained *Pseudomonas putida* strains with biodegradative potential. Appl. Environ. Microbiol. **64:**2073–2078.
38. **Morgan, A. F., and H. F. Dean.** 1985. Chromosomal map of *Pseudomonas putida* PPN, and a comparison of gene order with the *Pseudomonas aeruginosa* PAO chromosomal map. J. Gen. Microbiol. **131:**885–896.
39. **Moskovitz, J., M. A. Rahman, J. Strassman, S. O. Yancey, S. R. Kushner, N. Brot, and H Weissbach.** 1995. *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. J. Bacteriol. **177:**502–507.
40. **Mosqueda, G.** 1998. Personal communication.
41. **Nakazawa, T., and T. Yokota.** 1973. Benzoate metabolism in *Pseudomonas putida* (*arvilla*) mt-2: demonstration of two benzoate pathways. J. Bacteriol. **15:**262–267.
42. **Palleroni, N. J.** 1986. Section 4, family I, *Pseudomonadaceae*, p. 141–219. *In* P. H. A. Sneath, N. S. Mair, E. M. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
43. **Palmer, J. A., K. Hatter, and J. R. Sokatch.** 1991. Cloning and sequence analysis of the LPD-*glc* structural gene of *Pseudomonas putida*. J. Bacteriol. **173:**3109–3116.
44. **Pyle, L. E., and L. R. Finch.** 1988. A physical map of the genome of *Mycoplasma mycoides* subspecies *mycoides* Y with some functional loci. Nucleic Acids Res. **16:**6027–6039.
45. **Rainey, P. B.** 1998. Personal communication.
46. **Rainey, P. B., and M. J. Bailey.** 1996. Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. Mol. Microbiol. **19:**521–533.
47. **Ramos, C., L. Molina, and S. Vílchez.** 1998. Personal communication.
48. **Ramos, J. L., E. Díaz, D. Dowling, V. de Lorenzo, S. Molin, F. O'Gara, C. Ramos, and K. N. Timmis.** 1994. The behavior of bacteria designed for biodegradation. Bio/Technology **12:**1349–1356.
49. **Ramos, J. L., E. Duque, M. J. Huertas, and A. Haïdour.** 1995. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J. Bacteriol. **177:**3911–3916.
50. **Ramos, J. L., E. Duque, and A. Segura.** 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J. Bacteriol. **180:**3323–3329.
51. **Ramos, J. L., S. Marqués, and K. N. Timmis.** 1997. Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. Annu. Rev. Microbiol. **51:**341–373.
52. **Ramos, J. L., and K. N. Timmis.** 1987. Experimental evolution of catabolic pathways of bacteria. Microbiol. Sci. **4:**228–237.
53. **Ramos, J. L., A. Waserfallen, K. Rose, and K. N. Timmis.** 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235:**593–596.
54. **Ramos-González, M. I.** 1993. Liberación al medio ambiente de microorganismos manipulados genéticamente. Ph.D thesis. University of Granada, Granada, Spain.
55. **Ramos-González, M. I., and S. Molin.** 1998. Cloning, sequencing and phenotypic characterization of an *rpoS* gene from *Pseudomonas putida* KT2440. J. Bacteriol. **180:**3421–3431.
56. **Ramos-González, M. I., M. A. Ramos-Díaz, and J. L. Ramos.** 1994. Chromosomal gene capture mediated by the *Pseudomonas putida* TOL catabolic plasmid. J. Bacteriol. **176:**4635–4641.
57. **Ratnaningsih, E., S. Dharmsthiti, Y. Krishnapillai, A. Morgan, K. Sinclair, and B. W. Holloway.** 1990. A combined physical and genetic map of *Pseudomonas aeruginosa* PAO. J. Gen. Microbiol. **136:**2351–2356.
58. **Rech, S., U. Deppenmeier, and R. P. Gunsalus.** 1995. Regulation of the molybdate transport operon, *modABCD*, of *Escherichia coli* in response to molybdate availability. J. Bacteriol. **177:**1023–1029.
59. **Reddy, B. R., L. E. Shaw, J. R. Sayers, and P. A. Williams.** 1994. Two identical copies of IS*1246*, a 1275 base pair sequence related to other bacterial insertion sequences, enclose the *xyl* genes on TOL plasmid pWW0. Microbiology **140:**2305–2307.
60. **Reniero, D., J. J. Rodríguez-Herva, L. Molina, E. Galli, J. L. Ramos, and E. Duque.** Colonization of the corn root system by wild-type *Pseudomonas putida* KT2440 and mutants with altered surfaces. Submitted for publication.
61. **Rivera, E., L. Vila, and J. Barbé.** 1996. The *uvrB* gene of *Pseudomonas aeruginosa* is not DNA damage inducible. J. Bacteriol. **178:**5550–5554.
62. **Rivera, E., L. Vila, and J. Barbé.** 1997. Expression of the *Pseudomonas*

aeruginosa uvrA gene is constitutive. Mutat. Res. **377:**149–155.
63. **Robson, R. L., J. Chesshyre, C. Wheeler, R. Jones, P. Woodley, and J. R. Postgate.** 1984. Genome size and complexity in *Azotobacter chroococcum*. J. Gen. Microbiol. **130:**1603–1612.
64. **Rodríguez-Herva, J. J., M. I. Ramos-González, and J. L. Ramos.** 1996. The *Pseudomonas putida* peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the integrity of the cell envelope. J. Bacteriol. **178:**1699–1706.
65. **Römling, U., D. Grothues, W. Bautsch, and B. Tümmler.** 1989. A physical genome map of *Pseudomonas aeruginosa* PAO. EMBO J. **13:**4081–4089.
66. **Sambrook, J., E. F. Fritsch, and E. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
67. **Schurr, M. J., J. F. Vickrey, A. P. Kumar, A. L. Campbell, R. Cunin, R. C. Benjamin, M. S. Shanley, and G. A. O'Donovan.** 1995. Aspartate transcarbamoylase genes of *Pseudomonas putida*: requirement for an inactive dihydroorotase for assembly into the dodecameric holoenzyme. J. Bacteriol. **177:**1751–1759.
68. **Schwartz, D. C., W. Saffran, J. Welsh, R. Haas, M. Goldleuberg, and C. R. Canter.** 1982. New techniques for purifying large DNAs and studying their properties and packaging. Cold Spring Harbor Symp. Quant Biol. **47:**189–195.
69. **Segura, A.** 1998. Personal communication.
70. **Smith, C. L., S. R. Klco, and C. R. Cantor.** 1988. Pulsed-field gel electrophoresis and the technology of large DNA molecules, p. 41–72. *In* K. E. Davies (ed.), Genome analysis. IRL Press, Oxford, England.
71. **Strom, A. D., R. Hirst, J. Petering, and A. Morgan.** 1990. Isolation of high frequency of recombination donors from Tn*5* chromosomal mutants of *Pseudomonas putida* PPN and recalibration of the genetic map. Genetics **126:**497–503.
72. **Troup, B., M. Jahn, C. Hungerer, and D. Jahn.** 1994. Isolation of the *hemF* operon containing the gene for the *Escherichia coli* aerobic coproporphyrinogen III oxidase by in vivo complementation of a yeast HEM13 mutant. J. Bacteriol. **176:**673–680.
73. **Tümmler, B.** 1998. Personal communication.
74. **Watson, R. J.** 1990. Analysis of the C-4-dicarboxylate transport gene of *Rhizobium meliloti*: nucleotide sequence and deduced products of *dctA*, *dctB* and *dctD*. Mol. Plant-Microbe Interact. **3:**174–184.
75. **Wilkinson, S. R., D. I. Young, J. G. Morris, and M. Young.** 1995. Molecular genetics and the initiation of solvent to genesis in *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) NCIMB 8052. FEMS Microbiol. Rev. **17:**275–285.
76. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:**7–13.
77. **Yamamoto, S., and S. Harayama.** 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl. Environ. Microbiol. **61:**1104–1109.
78. **Yee, T. W., and D. W. Smith.** 1990. *Pseudomonas* chromosomal replication origins: a bacterial class distinct from *Escherichia coli*-type origins. Proc. Natl. Acad. Sci. USA **87:**1278–1282.
79. **Yim, L., and M. Vicente.** 1995. GenBank accession no. U29400.